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TITLE: Developing Novel Therapeutic Approaches in Small Cell Lung Carcinoma Using Genetically Engineered Mouse Models and Human Circulating Tumor Cells

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14. ABSTRACT
We have successfully developed mouse models in which to perform in vivo experiments, as well as developed expertise in live animal imaging to enable monitoring to tumors over time in these models. We have initiated treatment studies with chemotherapy and with targeted therapies in our models. Our preliminary data indicate that tumor response to chemotherapy in our models is modest. However, we find that tumor response to combination targeted therapy is significantly superior to either targeted therapy alone or to no treatment. Correlative biomarker studies are underway, including the isolation and enumeration of circulating tumor cells from SCLC patients. These findings support further development of this combination therapy approach and we anticipate ongoing experiments to assess correlative biomarkers.

15. SUBJECT TERMS
Small cell lung cancer (SCLC), Genetically engineered mouse model (GEMM), BH3 mimetic, TORC inhibitor, Apoptosis, Preclinical therapeutics

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Table of Contents

INTRODUCTION

Small cell lung cancer (SCLC) is an aggressive neuroendocrine carcinoma with a median survival of less than one year and a five-year overall survival of under 2% in the metastatic setting. While chemotherapy initially induces a response in most patients, metastatic disease invariably recurs rapidly and is often resistant to additional conventional therapies. To date, there are no effective targeted therapeutic approaches in SCLC and research efforts to develop new therapeutic strategies for these cancers have lagged far behind those for non-small cell lung cancer. This project aims to address these fundamental challenges by utilizing genetically engineered mouse models (GEMMs) of SCLC that faithfully recapitulate the human disease, as well as circulating tumor cells (CTCs) from both the GEMMs and human SCLC patients to develop a new therapeutic approach. Our therapeutic strategy, the combination of a BH3-mimetic and an mTOR complex (TORC) catalytic site inhibitor, is based on our understanding of the mechanisms of apoptosis and growth arrest in SCLC tumors. Our project aims to (1) explore the mechanism of chemotherapy response and resistance in the GEMM, (2) investigate the activity of combination BH3-mimetic and TORC inhibition therapy in the GEMM, and (3) utilize patient-derived CTCs to monitor treatment response. This report reviews our progress during the first year of funding.

KEYWORDS

Small cell lung cancer (SCLC)
Genetically engineered mouse model (GEMM)
BH3 mimetic
TORC inhibitor
Apoptosis
Preclinical therapeutics

ACCOMPLISHMENTS

Major goals of the project

Specific Aims:

Specific Aim 1: Determine biomarkers of response and resistance to standard chemotherapy in SCLC GEMMs.

Specific Aim 2: Perform preclinical study of combination targeted therapy in SCLC GEMMs.

Specific Aim 3: Utilize patient-derived CTCs as a means to monitor treatment response and predict sensitivity to treatment.

Major Tasks (as detailed in the Statement of Work):

Please see Appendix 1, Statement of Work, with proposed modifications incorporated. This is a collaborative project between Tyler Jacks's group at MIT (W81XWH-13-1-0323) and our group at MGH.

1. Generation of PR and PRP harboring SCLC tumors for use in chemotherapy studies.
2. Chemotherapy treatment studies in PR and PRP mice harboring SCLC tumors.
3. Collection and analysis of tissues from chemotherapy treated mice.
4. Collection and analysis of circulating tumor cells (CTCs) from chemotherapy treated mice.
5. Generation of PR and PRP mice for use in combination targeted therapy studies.
6. Acute treatment of mice with ABT-263, AZD8055, their combination, or vehicle and subsequent analysis
7. Chronic treatment of mice with ABT-263, AZD8055, their combination, or vehicle
8. Analyze CTCs from SCLC patients at MGH enrolled on DF/HCC protocol 05-300

Accomplishments under these goals

- **Major Task 1:** The first Aim of our project is designed to determine the biomarkers of response and resistance to standard chemotherapy in the SCLC GEMM model. This was performed by the Jacks group, as planned in the SOW.
- **Major Tasks 2 and 3:** The Jacks group performed optimization of radiographic imaging of animals, assessment of the pharmacodynamics of chemotherapy treatment in these models, and then measurement of response to chemotherapy treatment.

  The Jacks group developed an effective strategy incorporating both CT scanning and MRI to identify and measure tumors in PR mice. Most of these animals develop one measurable tumor. A representative example of a CT scan and corresponding MRI are shown in **Figure 1**. This figure highlights the fact that lung tumors are more easily measured using MRI imaging because of the relative absence of confounding adjacent tissue densities in the image. MRI imaging was used to measure tumors in PR mice, where mice develop on average one measurable tumor that can grow up to a size of roughly 8x8x8 mm before causing respiratory compromise. However, PRP mice develop roughly 20-30 radiographically evident tumors simultaneously. As a result, these tumors cannot grow to a size large enough for measurement by MRI without causing respiratory compromise in the animals. Therefore, they utilized micro-CT imaging to measure tumors in PRP mice.

  To validate that the chemotherapy treatments were active in these models, the Jacks laboratory identified tumor-bearing PRP mice using live-animal imaging (CT scanning and bioluminescence) and randomized animals to receive no treatment or cisplatin plus etoposide (7 mg/kg and 10 mg/kg, respectively, by intraperitoneal injection (IP)). Animals were sacrificed 24 hours after the treatment and lungs were inflated with 4% formaldehyde, fixed overnight in 4% formaldehyde, and then

paraffin embedded. Immunohistochemistry (IHC) was performed to detected phosphorylated H2AX, a marker of DNA damage. The number of P-H2AX nuclei were significantly higher in the cisplatin/etoposide treated tumors than in the untreated tumors (**Figure 2**). This result demonstrates that the chemotherapies used in these experiments cause DNA damage in autochthonous tumors in this model system.

To determine the effects of chemotherapy treatment on tumor growth in p53; Rb1 deleted tumors, tumor-bearing PR mice were randomized to receive treatment with cisplatin and etoposide (given as 7 mg/kg IP on days 1 and 8, and 10 mg/kg IP on days 2 and 9, respectively). Mice were imaged within one day prior to start treatment and at the end of the 28-day cycle. Tumor dimensions were measured and tumor volume fold change was determined. Within the first year of funding, 4 were in the chemotherapy arm and 5 animals in the control arm. There is no significant difference in the tumor volume fold change between the two groups (**Figure 3**). These studies are ongoing, and the Jacks laboratory is collecting tissue from treated animals as detailed in the statement of work, major task 3. Interestingly, early data suggest that there may be a bimodal distribution of responses in the treatment arm, with some tumors showing response to therapy and others not as is hinted by the existing data. If this is recapitulated in the larger data set, then we will follow up these results in an effort to identify differences between these two groups that may provide insights into the biology underlying chemotherapy sensitivity.

To determine the effects of chemotherapy treatment on tumor growth in p53; Rb1; Pten deleted tumors, tumor-bearing PRP mice were randomized to receive treatment with cisplatin and etoposide (given as 7 mg/kg IP on days 1 and 8, and 10 mg/kg IP on days 2 and 9, respectively). The Jacks laboratory used CT imaging to measure tumors and determine percent tumor volume change by comparing volumes of a representative tumor pre-treatment and 28 days after the initiation of treatment. Seven animals were included in the Cisplatin/Etoposide arm and ten animals were included in the untreated arm. There was no significant difference in tumor growth in the treated arm compared to the untreated arm (**Figure 4**).

- **Major Task 4:** The goal of this task is to collect circulating tumor cells from chemotherapy treated mice and analyze them for levels of BIM, Bcl-2, Bcl-xL and Mcl-1. Within our first two aims, we previously proposed that molecular studies of CTCs from the GEMMs would be performed using the Haber laboratory herringbone chip for CTC isolation. We have since optimized a more cost- and resource-efficient method of isolating CTCs from the GEMMs. (Please note that these preliminary experiments described here were proof of concept experiments not performed under the DOD-funded grant. These experiments were performed using other funding

mechanisms, and are shown here to demonstrate the feasibility of this approach.) To detect and isolate CTCs in animals harboring SCLC tumors, the Jacks laboratory has utilized PR animals that also harbor a Cre-activated tdTomato fluorescent reporter allele (*Rosa26::lox-stop-lox-tdTomato*; hereafter referred to as 'PRT') to fluorescently label tumor cells upon tumor initiation (Madisen et al., 2010). Whole blood is collected by terminal cardiac puncture from tumor-bearing PRT animals 12 months post-tumor initiation for analysis. The collected whole blood is subjected to red blood cell lysis, then sorted for fluorescent cells using fluorescence activated cell sorting (FACS). The number of CTCs collected ranged from 29 to over 70,000 per animal, in approximately 500 uL of collected blood (**Figure 5A**). Isolated CTCs stain positively for the neuroendocrine marker synaptophysin, validating their identity as SCLC tumor cells (**Figure 5B**). The Jacks laboratory will therefore perform CTC collection and analysis using this approach.

- **Major Task 5:** The goal of this task is to generate PR and PRP mice for use in combination targeted therapy studies. The Jacks laboratory has successfully completed infection of 130 PR mice needed to perform these studies. We had originally proposed to perform these targeted therapy studies in parallel in both PR and PRP mice. We had proposed these parallel studies to determine whether tumors lacking the Pten tumor suppressor gene would have different sensitivities to treatments than tumors that retained Pten. However, since then the Jacks laboratory has performed extensive whole exome and genome sequencing of PR SCLC GEMM tumors and have found that Pten is spontaneously lost in up to half of these tumors (McFadden et al., 2014). Based on this knowledge, we now feel that it would be redundant and inefficient to perform parallel treatment experiments in PR and PRP mice. Rather, we propose to focus our efforts exclusively on the PR mice. If we observe variability in response to treatments, then the Jacks laboratory can perform molecular analyses on tumors (including DNA sequencing, quantitative RT-PCR, and immunohistochemistry) to assess whether differential responses correlate with Pten status. <u>Therefore, we propose to eliminate further work with the PRP model within this portion of the proposal and instead direct all efforts to the PR model.</u>

- **Major Tasks 6 and 7:** The second Aim of our project is to explore the activity of the combination of treatment with the BCL-2 inhibitor ABT-263 and the TORC inhibitor AZD8055 in the SCLC GEMM model. Tumor-bearing SCLC GEMMs were randomized to receive either no treatment, AZD8055 alone (16mg/kg/qd), ABT-263 alone (80mg/kg/qd), or both ABT-263 and AZD8055, and were treated for 21 days (**Figure 6A**). Magnetic resonance imaging (MRI) of the thorax was performed one day prior to starting treatment and on day 21 of treatment, and lung tumor volumes pre- and post-treatment were quantified. Most animals had exactly one measurable

lung tumor. If more than one tumor was observed in separate lobes of the lungs, the larger tumor was measured. Tumors progressed in all untreated animals, though there was significant variability in the rate of progression over the 21-day period (**Figure 6B-D**). All three tumors treated with AZD8055 alone progressed (**Figure 6B, E, F**). Of three tumors treated with ABT-263, two progressed and one regressed (**Figure 6B, G, H**). By contrast, animals treated with combination ABT-263 and AZD8055 had significant regression or minimal progression of tumors (**Figure 6 B, I, J**). Consistent with these findings, cleaved caspase-3 (CC3) staining in allografted SCLC GEMM tumors was markedly apparent in the combination treatment at the three-day time-point (**Figure 6 K-N**), again indicating a strong apoptotic response following administration of this regimen. *Ex vivo* cell lines derived from SCLC GEMMs treated with AZD8055 had reduction of MCL-1 protein levels and were sensitized to ABT-263 (**Figure 7A and B**), consistent with the mechanism delineated in human cell lines and the activity observed in vivo.

- **Major Task 8:** The goal of this task is to analyze CTCs from patients with SCLC to assess for changes in CTC number and expression of markers relevant to our combination treatment strategy (P-4EBP1, P-S6, BIM, Bcl-2, Bcl-xL, and Mcl-1 using ISH and IHC) over the course of chemotherapy treatments. This work will be performed entirely at MGH within the Engelman laboratory. Our collaborators in the Haber laboratory at MGH are now using an improved microfluidic-based live CTC capture platform, the CTC-iChip (Karabacak et al., 2014; Ozkumur et al., 2013). Using antigen-based removal of leukocytes and granulocytes, this technology enables unbiased collection of unperturbed live CTCs in suspension. CTCs isolated by this method can also be fixed and stained after collection. We have begun to use the CTC-iChip technology to isolate viable SCLC CTCs and then perform the stains proposed above. We believe this will give higher quality results and more accurate assessment of expression of the markers of interest. In the first year of funding, we have made major strides towards accomplishing this task.
    1. We have incorporated this funding source into the Dana-Farber/Harvard Cancer Center (DF/HCC) protocol 05-300 to allow CTC collection, enumeration, and immunohistochemical analysis in SCLC patients. We have begun enrolling patients with SCLC onto this study, and have collected CTCs from 5 SCLC patients during the first year of funding. We developed a dual-color immunofluorescence stain to identify nucleated (DAPI-positive) SCLC cells that express tumor epithelial markers EGFR, EPCAM, CK8 and/or CK18 (labeled in FITC channel), but not hematopoietic markers CD45 or CD16 (labeled in TRITC channel), from patient samples enriched through the CTC-iChip, fixed onto glass slides, and imaged with automated fluorescence microscopy platform (BioView). From this initial patient cohort, we observed

evidence of CTCs in 3/5 samples, ranging from 10 to 112 CTCs per ml blood. Given the small patient number thus far, we cannot yet correlate CTC concentration with disease stage or prior therapies (Table 1), though we expect higher CTC burdens in extensive stage patients (Hiltermann et al., 2012). Examples of isolated CTCs are shown in Figure 8. We are on track with the target enrollment for this study (see Appendix A).

2. We are currently working to optimize staining techniques to assess levels of the proposed biomarkers.

Of note, generation of SCLC "CTC-derived xenografts" (CDXs) has recently been described (Hodgkinson et al., 2014). If CTC isolation via the CTC-iChip proves to be efficient, then generation of such xenografts from CTC product may be possible. This is a strategy that we hope to incorporate into this study in the future.

Opportunities for training and professional development provided by this project

N/A

How were the results disseminated to communities of interest?

- Poster Presentation: Farago AF et al., Application of a genetically engineered mouse model of small cell lung cancer for preclinical studies. Presented at the 14th Annual Targeted Therapies of the Treatment of Lung Cancer Meeting, sponsored by the International Association for the Study of Lung Cancer in Santa Monica CA, February 2014.
- Oral Presentation: Faber AC et al., Assessment of ABT-263 activity across a comprehensive cancer cell line collection leads to a novel, potent combination therapy for small cell lung cancer. Presented at the 2014 AACR Annual Meeting, San Diego CA, April 7, 2014.
- Publication: Assessment of ABT-263 activity across a cancer cell line collection leads to a potent combination therapy for small-cell lung cancer. Faber AC, Farago AF, Costa C, Dastur A, Gomez-Caraballo M, Robbins R, Wagner BL, Rideout WM 3rd, Jakubik CT, Ham J, Edelman EJ, Ebi H, Yeo AT, Hata AN, Song Y, Patel NU, March RJ, Tam AT, Milano RJ, Boisvert JL, Hicks MA, Elmiligy S, Malstrom SE, Rivera MN, Harada H, Windle BE, Ramaswamy S, Benes CH, Jacks T, Engelman JA. Proc Natl Acad Sci U S A. 2015 Mar 17;112(11):E1288-96. doi: 10.1073/pnas.1411848112. Epub 2015 Mar 3. PMID: 25737542.

What do you plan to do during the next reporting period to accomplish the goals?

- Biomarker assessment of tumor material from PR mice treated with chemotherapy, ABT-263, AZD8055, combination therapy, or no treatment.

- Ongoing CTC collection and analysis from human SCLC patients enrolled on DF/HCC protocol 05-300.
- Submission of a proposal and protocol to this funding agency to incorporate the generation of SCLC CTC-derived xenografts into this project.

IMPACT

What was the impact on the development of the principal discipline(s) of the project?

The data generated to date provide support for our combination therapy strategy in the treatment of SCLC. The data that we have collected so far show that in the genetically engineered mouse model of SCLC, combination of ABT-263 and AZD8055 causes tumor stabilization to tumor regression. With ongoing work on this combination, we are hopeful that these data will support movement of this therapeutic strategy into the clinic.

What is the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

CHANGES/PROBLEMS

Changes in approach and reasons for change

Our first two aims involve treatment studies in GEMMs of SCLC. The first aim is focused on chemotherapy treatments with cisplatin and etoposide, and the second is focused on treatments with ABT-263 (a BH3-mimetic) and AZD8055 (an mTOR catalytic inhibitor). We had originally proposed to perform these studies in parallel in two sets of genetically engineered mice: PR mice and PRP mice. We had proposed these parallel studies to determine whether tumors lacking the Pten tumor suppressor gene would have different sensitivities to treatments than tumors that retained Pten. However, since then the Jacks laboratory has performed extensive whole exome and genome sequencing of PR SCLC GEMM tumors and have found that Pten is

spontaneously lost in up to half of these tumors (McFadden et al., 2014). Based on this knowledge, we now feel that it would be redundant and inefficient to perform parallel treatment experiments in PR and PRP mice. Rather, we propose to focus our efforts exclusively on the PR mice. If we observe variability in response to treatments, then the Jacks laboratory can perform molecular analyses on tumors (including DNA sequencing, quantitative RT-PCR, and immunohistochemistry) to assess whether differential responses correlate with Pten status. Therefore, we propose to eliminate further work with the PRP model within this proposal and instead direct all efforts to the PR model.

Within our first two aims, we previously proposed that molecular studies of CTCs from the GEMMs would be performed using the Haber laboratory herringbone chip for CTC isolation. The Jacks laboratory has since optimized a more cost- and resource-efficient method of isolating CTCs from the GEMMs. To detect and isolate CTCs in animals harboring SCLC tumors, the Jacks laboratory has utilized PR animals that also harbor a Cre-activated tdTomato fluorescent reporter allele (*Rosa26::lox-stop-lox-tdTomato*; hereafter referred to as 'PRT') to fluorescently label tumor cells upon tumor initiation (Madisen et al., 2010). They collect whole blood by terminal cardiac puncture from tumor-bearing PRT animals 12 months post-tumor initiation for analysis. The collected whole blood is subjected to red blood cell lysis, then sorted for fluorescent cells using fluorescence activated cell sorting (FACS). The number of CTCs collected ranged from 29 to over 70,000 per animal, in approximately 500 uL of collected blood (Figure 5A). Isolated CTCs stain positively for the neuroendocrine marker synaptophysin, validating their identity as SCLC tumor cells (Figure 5B). We therefore propose to perform CTC collection and analysis in the Jacks lab using this approach. The PR mice being used in our studies already harbor a conditional tdTomato allele, so no additional or new crosses would be needed to generate the animals used for this approach.

For our third aim, we had proposed to perform quantification and molecular studies of CTCs derived from human SCLC patients before, during, and after treatment with chemotherapy. We had proposed to do this using the herringbone chip (Maheswaran et al., 2008; Nagrath et al., 2007; Yu et al., 2012). This technology captures cells to a device and allows for enumeration and IHC and in-situ hybridization (ISH) on CTCs, but does not enable collection or propagation of live CTCs. Recently, the Haber and Toner labs at MGH have developed a microfluidic-based live CTC capture platform, the CTC-iChip (Karabacak et al., 2014; Ozkumur et al., 2013). Using antigen-based removal of leukocytes and granulocytes, this technology enables unbiased collection of unperturbed live CTCs in suspension. The Haber lab has converted to doing most CTC isolation using this improved approach, and we believe this will provide higher quality assessment of biomarkers in CTCs. CTCs will be interrogated for relative

expression of TORC1 pathway mediators (P-S6, P-4EBP1) and apoptosis mediators (Bcl-2, Bcl-xL, BIM, Mcl-1) to determine if measurement of these molecular markers will be informative in clinical development of this novel therapy. Of note, we observed that the enumerated CTCs per mL is lower than what we would have expected based on previously published studies (Table 1). This may be because there are circulating malignant cells that lack epithelial markers (Figure 8), and instead may be better detected by a cocktail of antibodies also optimized for identification of neuroendocrine marker expression. We are therefore currently working on developing an approach for improved sensitivity of CTC identification and enumeration in SCLC.

Actual or anticipated problems or delays and actions or plans to resolve them

None

Changes that had a significant impact on expenditures

None. Work on the analysis of CTCs from patients with SCLC (Major Task 8) was delayed until June 2014. The final four months of the reporting period covers this work, and no members of the team other than the PI worked more than one person-month.

Significant changes in use or care of human subjects, vertebrate animals, biohazards and/or select agents

None

PRODUCTS

Publications, conference papers, and presentations

- Poster Presentation: Farago AF et al., Application of a genetically engineered mouse model of small cell lung cancer for preclinical studies. Presented at the 14th Annual Targeted Therapies of the Treatment of Lung Cancer Meeting, sponsored by the International Association for the Study of Lung Cancer in Santa Monica CA, February 2014.
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Benes CH, Jacks T, Engelman JA. Proc Natl Acad Sci U S A. 2015 Mar 17;112(11):E1288-96. doi: 10.1073/pnas.1411848112. Epub 2015 Mar 3. PMID: 25737542. See Appendix.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	Jeffrey Engelman
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	No change
Nearest person month worked:	1
Contribution to Project:	
Funding Support:	

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - Nothing to report
- **What other organizations were involved as partners?**
  - Nothing to Report.

2. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:** For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ers.amedd.army.mil for each unique award.
- **QUAD CHARTS:** If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

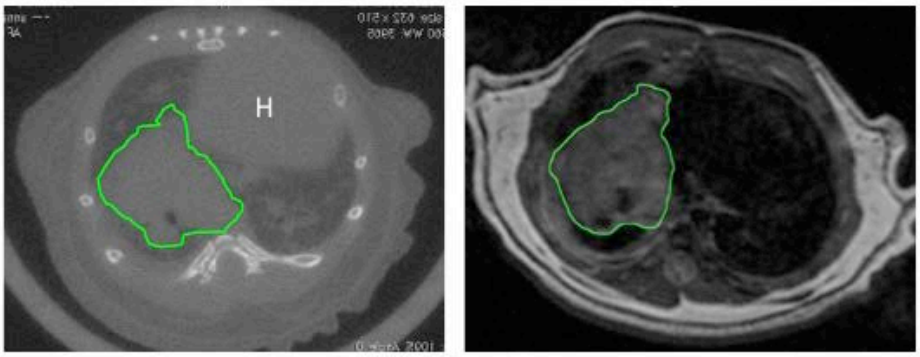


Figure 1. Micro CT and MRI are used to identify and measure tumors in the SCLC GEMMs. Representative axial micro-CT (left) and MRI (right) images in a PR mouse are shown. The lung tumor is outlined in green. The heart is seen in the micro-CT image only and is marked (H).

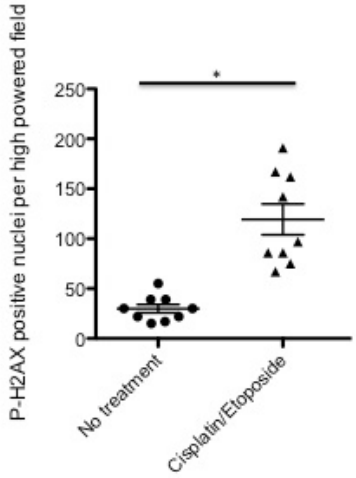


Figure 2. Assessment of chemotherapy activity in autochthonous PRP tumors in 24 hour treatment. Two to three animals were included in each group, with three tumors in each animal included in analysis. Black lines represent mean and standard error of the mean (SEM). * $p<0.0001$ in two-tailed t test.

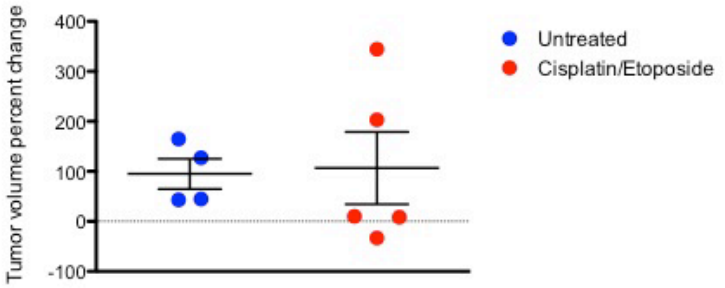


Figure 3. Effect of chemotherapy treatment on PR tumor volume over 28 days. Tumor bearing PR mice were treated with cisplatin (7 mg/kg IP days 1 and 8) and etoposide (10 mg/kg IP days 2 and 9), and were imaged by MRI within one day prior to treatment and then 28 days later. Tumor volumes were measured using Osirix software and percent volume change is plotted. Black lines and error bars represent mean and standard error of the mean (SEM). These results are not statistically significantly different using a two-tailed t test (p=0.8958).

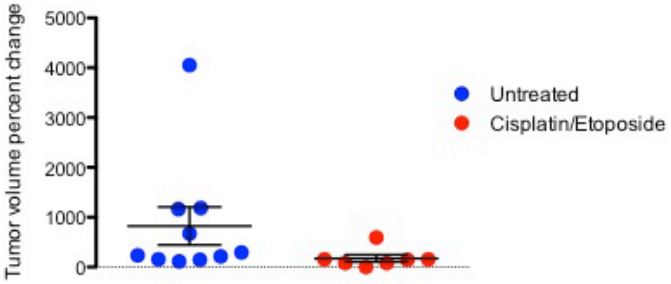
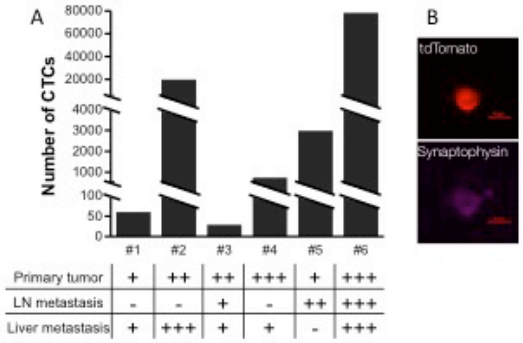


Figure 4. Effect of chemotherapy treatment on PRP tumor volume over 28 days. Tumor bearing PRP mice were treated with cisplatin (7 mg/kg IP days 1 and 8) and etoposide (10 mg/kg IP days 2 and 9), and were imaged by CT within one day prior to treatment and then 28 days later. For each animal, one representative tumor was measured to determine volume using Osirix software and percent volume change is plotted. Black line and error bars represent mean and SEM, respectively. These results are not statistically significantly different using a two-tailed t test (p= 0.1827).



	#1	#2	#3	#4	#5	#6
Primary tumor	+	++	++	+++	+	+++
LN metastasis	-	-	+	-	++	+++
Liver metastasis	+	+++	+	+	-	+++

Figure 5: Detection of fluorescently-labeled CTCs from whole blood in PRT tumor-bearing animals. (A) CTC counts from six tumor-bearing PRT animals are shown, as detected and quantified by flow cytometry. Corresponding primary tumor and metastatic burdens were estimated based on visual inspection of dissected whole lungs, lymph nodes and liver for tdTomato fluorescence. (B) CTCs were plated and stained for synaptophysin.

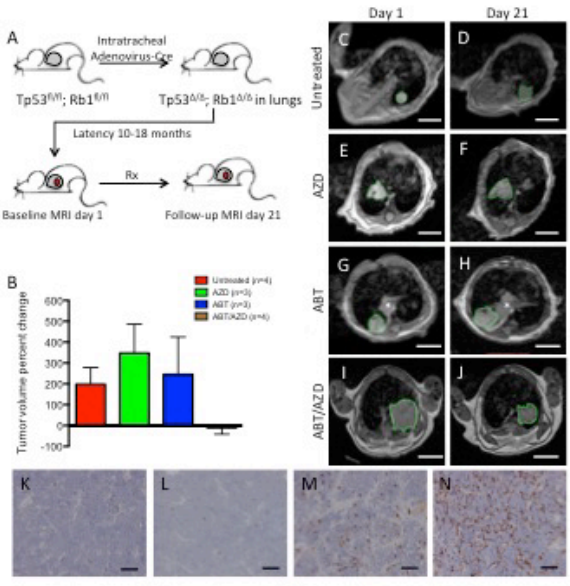


Figure 6: Combination ABT-263 and AZD8055 induce tumor regressions in a GEMM of SCLC. (A) Schematic of GEMM experiment. Gray space in mouse drawing indicates lung, red circle indicates lung tumor. (B) SCLC GEMMs harboring radiographically measurable lung tumors were treated for 21 days with 80 mg/kg/qd ABT-263, 16 mg/kg/qd AZD8055, combination ABT-263/AZD8055 or were untreated. Chest and upper abdomen were imaged by MRI on days 1 and 21 of treatment, and for each animal the largest single lung tumor was measured and volume was computed. The mean percent tumor volume change is plotted for each group with bars indicating standard error of the mean. The number of mice per group (n) is indicated. Unpaired one-tailed t-test for statistical significance comparing tumor volume percent change between pairs of datasets: untreated vs ABT-263/AZD8055: p=0.0255. AZD8055 vs ABT-263/AZD8055: p=0.0330. ABT-263 vs ABT-263/AZD8055: p= 0.0800. (C-J) Representative examples of MRI images (with tumors outlined in green) on day 1 and day 21 of a 21-day treatment period, with the treatment indicated. * in G and H indicates additional tumor in separate lobe, not included in measurements. Scale bar: 5.0 mm. (K-N) A GEMM SCLC tumor was dissected from the lung and implanted subcutaneously into a NOD *scid* gamma (NSG) mouse. An established tumor in the NSG mouse was then divided and subcutaneously implanted directly into nu/nu four NSG mice. There was no *in vitro* intermediate. Once tumors were established, nu/nu mice were treated for three days with (K) vehicle for both drugs, (L) AZD8055 (16 mg/kg/qd), (M) ABT-263 (80 mg/kg/qd), (N) ABT-263 (80 mg/kg PO daily) and AZD8055 (16 mg/kg PO daily). Tumors were collected and fixed three hours after the final treatment. Sections from tumor were stained to detected cleaved caspase 3, shown as brown stain. Scale bar: 100 um.

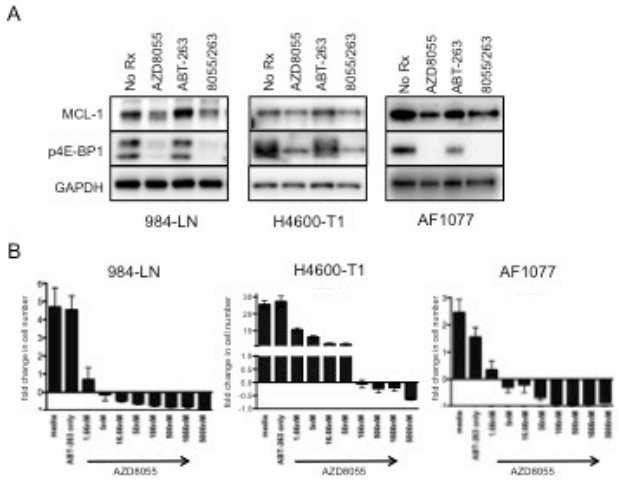


Figure 7. Cell lines established from a SCLC GEMM downregulate MCL-1 following AZD8055 treatment and are sensitized to ABT-263 by the addition of AZD8055. (A) The three indicated murine SCLC cell lines (984-LN, H4600-T1, and AF1077) were treated with no drug (control), 500 nM AZD8055, 1 μM ABT-263, or combination 500 nM AZD8055/1 μM ABT-263 and after 16 h of treatment, lysates were probed with the indicated antibodies. (B) Murine SCLC cell lines were treated with no drug (Media), 100 nM ABT-263 (ABT-263 only), or 100 nM ABT-263 in combination with the indicated dose of AZD8055 (ranging from 1.66 nM to 5000 nM AZD8055) for seven days. The number of viable cells was determined by CellTiter-Glo® and presented as fold-change of cells compared to day 0 (i.e., negative values represent loss of cells from day 0). Error bars are S.E.M. of cells treated in triplicate.

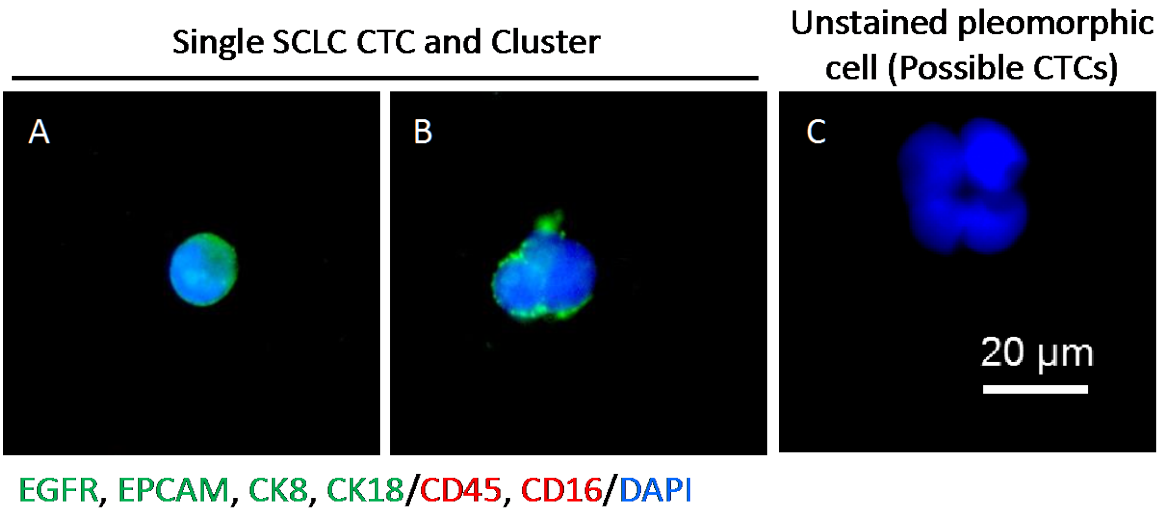


Figure 8: CTCs from SCLC patient. Cells were isolated from whole blood using the CTC iChip-neg technique. (A) Single cell (DAPI positive nucleus, blue) staining positive for epithelial markers (green). (B) Cluster of two cells staining positive for epithelial markers. (C) Cluster of cells with pleomorphic appearance but staining negative for epithelial markers. These are suspected malignant cells. All cells shown stained negative for leukocyte markers (red).

05-300 Patient ID	Diagnosis	Stage at collection	Prior Therapies	CTCs per mL of whole blood
SL-01	SCLC	ES	none	10
SL-02	SCLC	ES	none	<5
SL-03	Mixed SCLC and LCNEC	ES	none	112
SL-05	SCLC	LS	none	0
SL-06	SCLC	ES	Platinum doublet chemotherapy	0

Table 1: Enumeration of CTCs from SCLC patients. Consenting patients with small cell lung cancer (SCLC) receiving treatment at MGH were accrued for this study according to DF/HCC protocol 05-300. Circulating tumor cells (CTCs) were isolated from 10-20 mL whole blood using the ^{neg}CTC-iChip. Enriched CTCs were spun onto glass slides for enumeration by immunofluorescence. Nucleated (DAPI-positive) cells positive for epithelial markers (EGFR, EPCAM, CK8, CK18) and negative for hematopoietic markers (CD45, CD16) were scored as CTCs. Presented in the table are pathologic diagnosis, stage, therapies received prior to CTC collection, and CTCs per mL whole blood. Note that patient SL-04 failed peripheral blood collection. LCNE: Large cell neuroendocrine carcinoma. LS: Limited stage. ES: Extensive stage.

APPENDICES

1. Statement of Work, with proposed modifications included. Please note that this was submitted **9/22/14** to the Department of Defense for review.

2. Published manuscript: Publication: Assessment of ABT-263 activity across a cancer cell line collection leads to a potent combination therapy for small-cell lung cancer.
   Faber AC, Farago AF, Costa C, Dastur A, Gomez-Caraballo M, Robbins R, Wagner BL, Rideout WM 3rd, Jakubik CT, Ham J, Edelman EJ, Ebi H, Yeo AT, Hata AN, Song Y, Patel NU, March RJ, Tam AT, Milano RJ, Boisvert JL, Hicks MA, Elmiligy S, Malstrom SE, Rivera MN, Harada H, Windle BE, Ramaswamy S, Benes CH, Jacks T, Engelman JA. Proc Natl Acad Sci U S A. 2015 Mar 17;112(11):E1288-96. doi: 10.1073/pnas.1411848112. Epub 2015 Mar 3. PMID: 25737542.

APPENDIX 1: Statement of Work, with proposed modifications included. Please note that this was submitted **9/22/14** to the Department of Defense for review.

STATEMENT OF WORK – 07/01/2013
PROPOSED START DATE September 1, 2013

Site 1: Massachusetts Institute of Technology
Koch Institute for Integrative Cancer Research

500 Main Street

Cambridge MA 02139

Initiating PI: Dr. Tyler Jacks

Site 2: Massachusetts General Hospital
Cancer Center

Building 149

13th Street

Charlestown MA 02129

Partnering PI: Dr. Jeffrey Engelman

Specific Aim 1: Determine biomarkers of response and resistance to standard chemotherapy in SCLC GEMMs.	**Timeline**	**Site 1** (Initiating PI)	**Site 2** (Partnering PI)
Major Task 1: Generation of PR and PRP harboring SCLC tumors for use in chemotherapy studies	Months		
Subtask 1: Infection of PR mice (age 6-8 weeks) with adenovirus-Cre. • Number of mice to infect: 60 **COMPLETED**	1-6	Dr. Jacks	
Subtask 2: Infection of PRP mice (age 6-8 weeks) with adenovirus-Cre. • Number of mice to infect: 60 **UNDERWAY** MODIFICATION: We will discontinue infection of PRP mice for this project.	1-9	Dr. Jacks	
Major Task 2: Chemotherapy treatment studies in PR and PRP mice harboring SCLC tumors. Modification: Going forward, we propose to limit chemotherapy treatment studies to PR mice.			
Subtask 1: MRI/CT imaging and acute treatment • PRP mice (months 5-8) **COMPLETED** • PR mice (months 10-14) **UNDERWAY**	5-14	Dr. Jacks	

Subtask 2: Luminescence imaging, MRI/CT imaging, and chronic treatment of mice • PRP mice (months 5-12) **PENDING** • PR mice (months 10-18) **UNDERWAY** MODIFICATION: We will continue chronic chemotherapy experiments in PR mice and will discontinue chronic chemotherapy experiments in PRP mice.	5-12	Dr. Jacks	
Major Task 3: Collection and analysis of tissues from chemotherapy treated mice. Modification: These analyses will be conducted primarily in PR mouse tissue.			
Subtask 1: Histologic analysis of acutely and chronically treated mice, including IHC for markers of platinum activity and DNA damage (months 6-18) **UNDERWAY**	6-18	Dr. Jacks	
Subtask 2: Cell line derivation from chronically treated mice (months 9-18) **UNDERWAY**	9-18	Dr. Jacks	
Subtask 3: Protein analysis of tumors from acutely and chronically chemotherapy-treated mice. • Jacks lab: Collection of frozen tissue and preparation of protein lysate. • Engelman lab: IHC and Western blotting for Bcl-2, BIM, Mcl-1, Bcl-xL, and PUMA • Engelman lab: IP for BIM and blotting for associated proteins **UNDERWAY**	9-18	Dr. Jacks	Dr. Engelman
Subtask 4: Identification of differentially expressed transcripts in chemotherapy sensitive vs resistant tumors. • Preparation of total RNA and submission to biopolymers facility for mRNA sequencing (months 12-18) • Bioinformatic analysis of data (months 18-24)	12-24	Dr. Jacks	
Major Task 4: Collection and analysis of circulating tumor cells (CTCs) from chemotherapy treated mice.			
Subtask 1: Collect whole blood from mice at time of necropsy **UNDERWAY**	6-18	Dr. Jacks	
Subtask 2: Run blood samples on Epcam-based CTC capture chips, perform IHC and ISH for BIM, Bcl-2, Bcl-xL, and Mcl-1 **MODIFICATION**: CTC collection will be performed in the Jacks laboratory using fluorescence activated cell sorting (FACS) to identify TdTomato-expressing tumor cells in whole mouse	6-18	Dr. Jacks	~~Dr. Engelman~~

blood.			
Milestone #1: *Co-author manuscript describing the efficacy of standard chemotherapy in the PR and PRP models, with emphasis on differences between PR and PRP chemosensitivity, apoptotic response to chemotherapy in the acute versus chronic setting, and expression differences between untreated, acutely treated, and chronically treated tumors*	18-24	Dr. Jacks	Dr. Engelman
Specific Aim 2: Perform preclinical study of combination targeted therapy in SCLC GEMMs.			
Major Task 5: Generation of PR and PRP mice for use in combination targeted therapy studies. Modification: Going forward, we propose to limit combination targeted therapy treatment studies to PR mice.			
Subtask 1: Infection of PR mice (age 6-8 weeks) with adenovirus-Cre. • Number of mice to infect: 130 **UNDERWAY**	1-9	Dr. Jacks	
Subtask 2: Infection of PRP mice (age 6-8 weeks) with adenovirus-Cre. • Number of mice to infect: 130 **PENDING** Modification: We will discontinue infection of PRP mice.	1-12	Dr. Jacks	
Major Task 6: Acute treatment of mice with ABT-263, AZD8055, their combination, or vehicle and subsequent analysis			
Subtask 1: Acute treatments of mice and tissue collection • MRI/CT imaging and acute treatments of PR and PRP mice • Collection of tumors for histology and protein lysate preparation **UNDERWAY** in PR mice	12-18	Dr. Jacks	
Subtask 2: IHC and western blotting of tumors for P-4EBP1, P-S6, BIM, Bcl-2, Bcl-xL, Mcl-1 **UNDERWAY** from PR tumors	12-30		Dr. Engelman
Subtask 3: CTC capture and analysis • Jacks lab: Collection of whole blood for CTC capture • Engelman lab: Run blood samples on Epcam-based CTC capture chips, perform IHC and ISH for P-4EBP1, P-S6, BIM, Bcl-2, Bcl-xL, Mcl-1 **PENDING** **MODIFICATION**: CTC collection will be performed in the Jacks laboratory using fluorescence activated cell sorting (FACS) to identify TdTomato-expressing tumor cells in whole mouse	12-24	Dr. Jacks	Dr. Engelman

blood.			
Major Task 7: Chronic treatment of mice with ABT-263, AZD8055, their combination, or vehicle			
Subtask 1: Imaging by luminescence and MRI, and treatment of chemotherapy-naïve mice with targeted therapy **UNDERWAY** in PR mice	18-30	Dr. Jacks	
Subtask 2: Treatment of chemotherapy-resistance mice with targeted therapy	21-30	Dr. Jacks	
Milestone #2: *Co-author manuscript describing efficacy of novel combination targeted therapy ABT-263/AZD8055 in genetically distinct models of SCLC (PRP and PR) and in chemotherapy-resistant mSCLC tumors.*	30-32	Dr. Jacks	Dr. Engelman
Specific Aim 3: Utilize patient-derived CTCs as a means to monitor treatment response and predict sensitivity to treatment.			
Major Task 8: Analyze CTCs from SCLC patients at MGH enrolled on DF/HCC protocol 05-300 **MODIFICATION:** CTCs will be collected using the CTC-iChip technology and directly transplanted into immune compromised mice to generate xenograft models.			
Subtask 1: Enroll patients and collect CTCs. Quarterly enrollment goal: 4 to 5 patients. Overall enrollment goal: 40 patients. **UNDERWAY** **MODIFICATION: overall enrollment goal 20 patients**	6-33		Dr. Engelman
Subtask 2: Analyze CTC number and correlate with radiographic changes in disease CTC quantification is underway	12-36		Dr. Engelman
Subtask 3: Analyze CTCs for P-4EBP1, P-S6, BIM, Bcl-2, Bcl-xL, and Mcl-1 using ISH and IHC **PENDING**	12-33		Dr. Engelman
Milestone #3*: Co-author manuscript describing the utility of human SCLC CTC measurement and analysis for tracking the progression of disease and predicting sensitivity to treatments, with correlation to findings in the mouse models.*	33-36	Dr. Jacks	Dr. Engelman

Projected Quarterly Enrollment for CTC collection under MGH protocol 05-300

	Year 1				Year 2							
Target Enrollment (per quarter)	**Q1**	**Q2**	**Q3**	**Q4**	**Q1**	**Q2**	**Q3**	**Q4**	**Q1**	**Q2**	**Q3**	**Q4**
Site 2: MGH (original)			4	4	4	4	4	5	5	5	5	
MODIFIED target enrollment:			**2**	**2**	**2**	**2**	**2**	**2**	**2**	**3**	**3**	
Target Enrollment (cumulative) (original)			**4**	**8**	**12**	**16**	**20**	**25**	**30**	**35**	**40**	
MODIFIED total enrollment (cumulative)			**2**	**4**	**6**	**8**	**10**	**12**	**14**	**17**	**20**	